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Melissa Encinias

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Reproductive Character Displacement in *Calopteryx aequabilis* and *C. maculata*:  
Improving Species Recognition Through the Divergence of Male Mating Preferences

Melissa Encinias

A thesis submitted to the Graduate Faculty of

JAMES MADISON UNIVERSITY

In

Partial Fulfillment of the Requirements

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## Dedication Page

This thesis is dedicated to my parents.

## Acknowledgments

I would like to thank my advisor, Dr. Idelle Cooper, and my committee members, Dr. Heather Griscom and Dr. David McLeod, for all their guidance, motivation, and support.

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## Abstract

An ongoing evolutionary question is how co-occurring species maintain reproductive barriers when they are morphologically, behaviorally, and ecologically similar. Without geographic isolation, traits involved in species recognition may be under selection to enhance reproductive barriers. Exaggerated trait differences between species in sympatric populations may reflect selection to reduce misdirected mating between species, or reproductive character displacement. While this phenomenon is widely recognized as an important stage in the speciation process, there is little direct evidence of this process in nature. In two North American damselfly species, *Calopteryx aequabilis* and *C. maculata*, wing pigmentation is sexually dimorphic and also shows exaggerated differences in sympatric populations, particularly in female wings. When these species occur together, they occupy the same mating territories and the potential for misdirected mating is high. I hypothesize that female wing pigmentation is under selection for species recognition. In this study, I conducted male mate choice experiments in which I altered female wings by switching them within and between species. I measured male mating preference of both species in allopatric and sympatric populations by giving males a choice of two female types in a natural setting. Results supported the hypothesis that male preferences in sympatry diverge corresponding to female wing pigmentation. Sympatric *C. aequabilis* males preferred lighter-winged females, which significantly differed from the dark wing preferences of *C. maculata* males and allopatric *C. aequabilis* males. By manipulating the female wing pigmentation directly, I identified that this is the specific trait under selection. These findings indicate that male mating preferences and female wing pigmentation diverged in sympatry to reduce misdirected mating of two closely related species.



## Introduction

Understanding how species achieve reproductive isolation is a major evolutionary question that will help us understand the origin of biodiversity, including variation within and between species. Isolation naturally occurs when species are geographically separated or have allopatric populations (Dobzhansky, 1940), but how reproductive barriers evolve in sympatric, or co-occurring, populations are more difficult to discern. Like ecological character displacement, when species compete for ecological resources, reproductive character displacement may occur when sympatric species interfere in reproductive processes. Character displacement can result in competitive exclusion (Levin, 1970; Losos, 2000), the two species hybridizing into one species (Webb *et al.* 2011; Sánchez-Guillén *et al.* 2014), or divergence of reproductive traits between species (Pfenning & Pfenning, 2009). If mismatched courtship and matings are costly or if hybrid offspring have lower fitness (e.g. being inviable, infertile, unhealthy, or unattractive), reproductive barriers can evolve that prevent heterospecific matings through enhanced species recognition (Dobzhansky, 1940; Coyne & Orr, 1998; Cutter, 2012; Garner *et al.* 2018).

Mate choice can lead to reproductive isolation if differential mating preferences, or sexual selection pressures, act as a form of species recognition (Ridgeway and McPhail, 1984; Heisler *et al.* 1987; Friberg *et al.* 2008). Understanding the reproductive interactions between sympatric species can provide a better understanding of how species coexist and speciate (Pfenning and Pfenning, 2009). However, determining how individuals recognize conspecifics and select a mate is not always apparent, as mating preferences can be difficult to detect (Kokko *et al.* 2003).

One system that can offer a unique insight to the role of mate choice as a form of prezygotic reproductive isolation is the Jewelwing damselfly radiation of North America, which includes *Calopteryx aequabilis* and *C. maculata*. These species have both allopatric and sympatric populations, with the species distribution of *C. aequabilis* ranging from the northern US to northern Canada, and *C. maculata* spanning the eastern half of the contiguous US (Hassall, 2014; Iyengar *et al.*, 2014; Waage, 1975). When these species occur in sympatry, divergence of wing color has been observed in females, but not males (Waage, 1979). Both allopatric *C. aequabilis* and *C. maculata* females have

dark, highly pigmented wings. However, sympatric *C. aequabilis* females have significantly lighter wing color than both allopatric *C. aequabilis* and sympatric *C. maculata* females (Waage, 1979). This enhancement of phenotypic differences between co-occurring species suggests that wing pigmentation is under selection during mate choice as a reproductive barrier (Brown and Wilson, 1956; Iyengar *et al.*, 2014).

Wing pigmentation plays an important role in both sexual selection and species recognition in the Caloptergidae genus (Svensson *et al.* 2006; Serrano-Meneses *et al.* 2008; Svensson and Waller, 2013), including *C. aequabilis* and *C. maculata*. In this system, females ready to reproduce will perch along streams, waiting for males to approach. Then, the males will court the females using elaborate wings displays (Meek & Herman, 1990). Previous research has focused almost exclusively on selection on male damselflies (Meek & Herman, 1990; Siva-Jothy, 1999; Córdoba-Aguilar, 2002; Svensson *et al.* 2006; Serrano-Meneses *et al.* 2008; Svensson and Waller, 2013). However, the role of sexual selection in females may also play an important role, because like males, females vary in degrees of mating success and have similar selection pressures (Clutton-Brock & Huchard, 2013; Allison, 2016). Therefore, it is possible that this observed divergence in female wing color is functioning as a reproductive barrier in sympatry.

Species recognition is likely the cause of *Calopteryx* female wing color divergence as no other barriers (behavioral, ecological, mechanical, geographic, or temporal) have been observed in this system (Waage, 1975). Additionally, heterospecific mating is rarely observed though hybridization is possible (Waage, 1975). Research by Waage (1975) supported the idea of female wing color acting as a reproductive barrier by showing that both allopatric and sympatric *C. maculata* males preferred dark-winged over light-winged females. However, that study did not account for female mating behaviors, as dead female models were utilized, and *C. aequabilis* male mating preferences were also not determined. I aimed, therefore, to determine if the mating preferences of both sympatric *C. aequabilis* and *C. maculata* males differ between allopatric and sympatric populations, and if mate choice is affected by female wing pigmentation. I hypothesized that males of both species would prefer the pigmentation of sympatric, conspecific females, and that male preference of the two species would diverge in sympatric populations. To test this hypothesis, I conducted mate choice

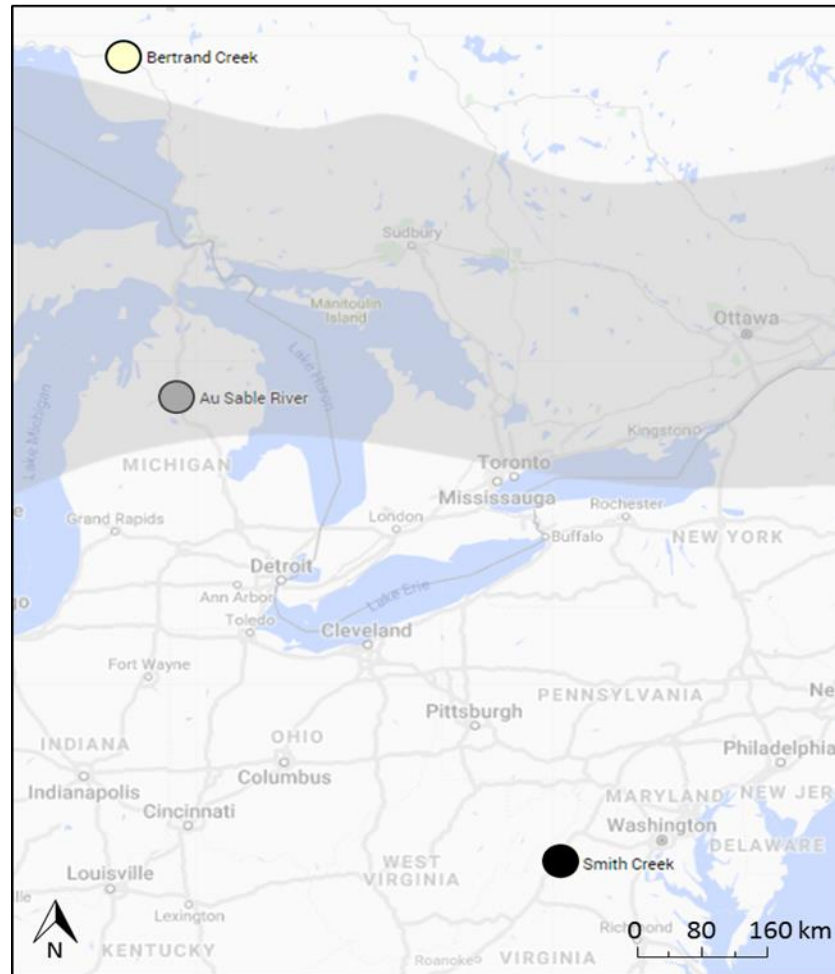
experiments in which I manipulated female wing pigmentation and measured *Calopteryx* male preference of dark- or light-winged females in sympatric and allopatric populations.

## Methods

To determine male preferences of female *Calopteryx* wing color, I conducted two types of mate choice experiments between 6/18/18 and 7/21/18: tent and tether experiments. These experiments were designed to give *C. aequabilis* and *C. maculata* males a choice of either dark- or light-winged females that were surgically altered in both species. Tent experiments measured sympatric male preferences only, and tether experiments measured both sympatric and allopatric male preferences. Additionally, I surveyed population ratios at the sympatric experimental site to better understand the local composition of co-occurring *Calopteryx* populations and verify that sympatric species do indeed have the possibility to interact and hybridize at a smaller spatial scale.

### *Damselfly Collections*

Female *Calopteryx* damselflies were collected throughout the experimental period to provide a supply of wings for the mate choice experiments. Allopatric *C. aequabilis* were collected from Bertrand Creek, Ontario (48°42'39.9"N, 85°33'43.3"W; Figure 1) and allopatric *C. maculata* from Smith Creek, VA (38°37'38.6"N, 78°39'45.8"W; Figure 1). Sympatric *Calopteryx* damselflies of both species were collected in the Au Sable River, MI (44°39'29.6"N, 84°43'45.0"W; Figure 1). All damselflies were collected using insect nets (40cm-diameter hoop, aerial net, aluminum handle). To minimize handling effects, collectors removed bug spray and/or sunscreen from their hands before collections. Each specimen collected for wings was measured for wing length and frozen in individual glassine envelopes until used for the wing transplant experiments.

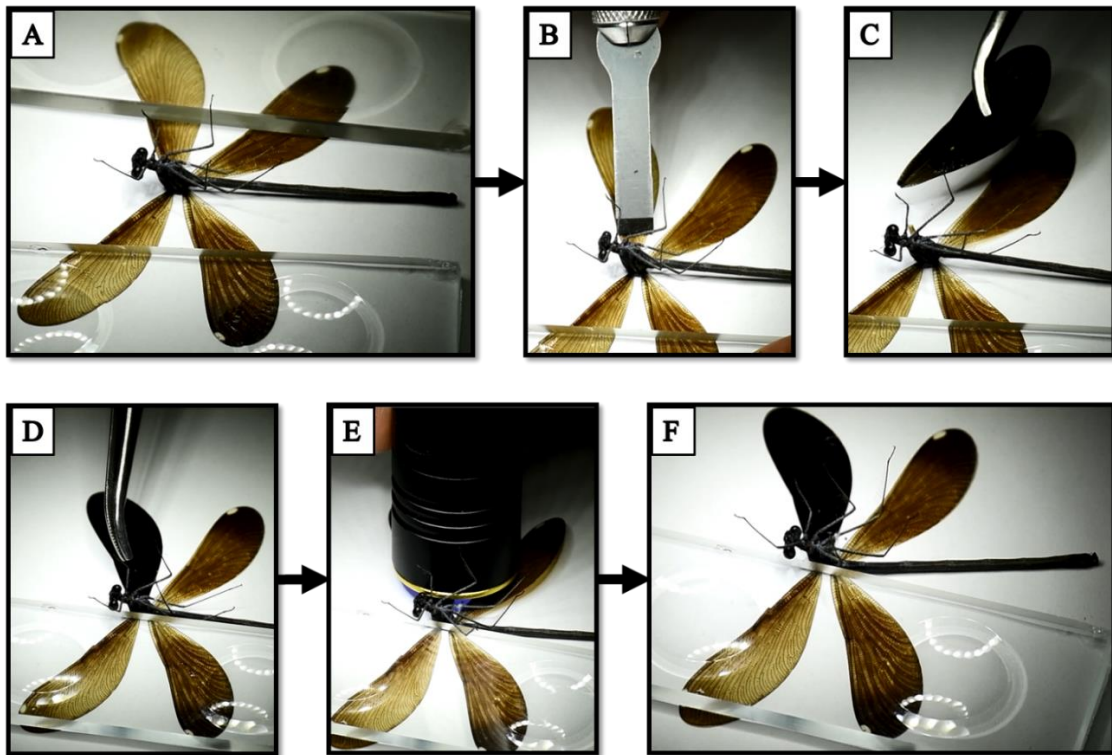


**Figure 1.** Locations of the mate choice experiments conducted on *Calopteryx* populations. Allopatric *C. aequabilis* populations were at Bertrand Creek, Ontario (yellow), allopatric *C. maculata* populations were at Smith Creek, Virginia (black), and sympatric populations were located along the Au Sable River, Michigan (grey). Grey shaded region indicates the area of sympatry from other studies (Cooper et al., unpublished data).

### *Wing Grafting Procedure*

To control for other differences between species, we experimentally manipulated female damselflies by removing and replacing their wings with a new set of the same approximate size. We followed the general methods of Greene et al. (1987) that transplanted wings between *Musca domestica* and *Zonosemata* flies. In this study, however, we used thin Loon Outdoors UV Clear Fly Finish glue and a Loon Outdoor UV Bench Light to cure the glue.

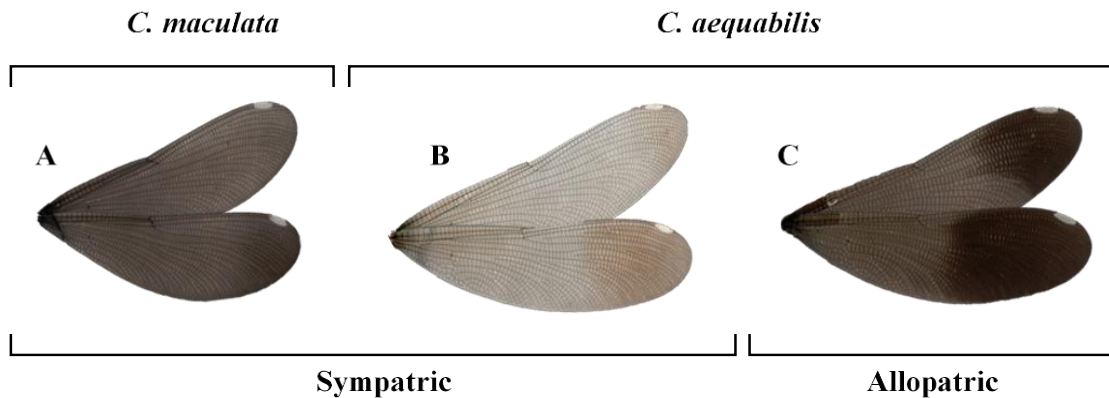
All females being manipulated (*i.e.* receiving wing grafts) for tent and tether experiments were collected during the day prior to the procedure. The damselflies were kept overnight in mesh-net cages covered in a damp cloth towel and placed in a cooler with ice. For the procedure, the wings of these females were removed and replaced with frozen wings using the wing grafting procedure depicted below (Figure 2; Appendix). After the wing grafts, females were still able to fly and mate for up to two days before the wings became brittle.



**Figure 2.** Damselfly wing manipulation procedure. (A) The damselfly is placed on her back with weights (*e.g.* standard microscope slide) on her wings to hold her in place. (B) The original wing is cut and removed with a Stanley razor blade or x-acto knife, leaving a small portion of the wing near the thorax. (C – D) The new wing, with glue on the tip, is placed on the remaining portion of the original wing. (E) UV curing light applied for 20 seconds as close to the wing as possible to avoid exposing the female to UV light. (F) Completed wing transplant of the female's left forewing. In the example shown, a male *C. maculata* wing is transplanted onto a female *C. maculata* for the purpose of showing color contrast. In experimental manipulations, females only received wings from other females.

### *Tent Experiments*

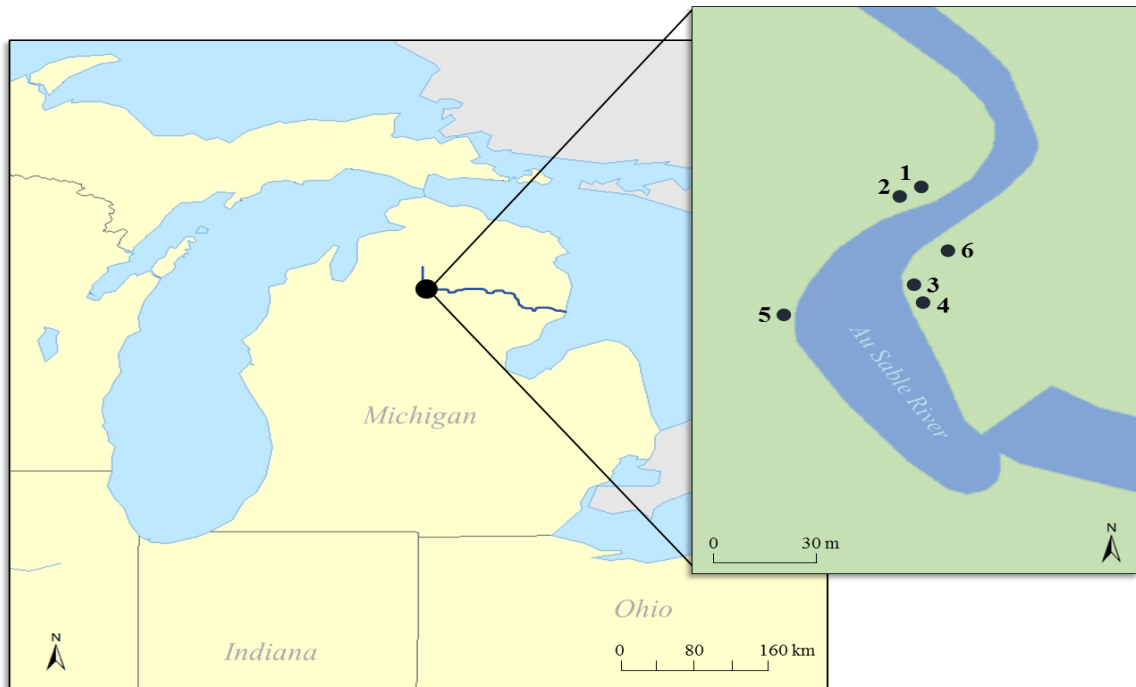
To determine the mating preferences of sympatric *Calopteryx* males, we conducted tent experiments along the Au Sable River in Grayling, MI (5973 W M-72 Hwy, Grayling, MI 49738). Sympatric *Calopteryx* males were given a choice of dark- and light-winged females (Figure 3). Sympatric *C. aequabilis* males were presented with unmanipulated sympatric *C. aequabilis* and *C. maculata* females. No additional trials were performed with *C. aequabilis* males due to the lack of mating attempts. We conducted five experimental treatments with Sympatric *C. maculata* males: 1. unmanipulated sympatric *C. aequabilis* x *C. maculata* females; 2. sympatric *C. aequabilis* x *C. maculata* females with heterospecific wings; 3. sympatric *C. maculata* females, with either allopatric or sympatric *C. aequabilis* wings; 4. sympatric *C. aequabilis* females, with either allopatric or sympatric *C. aequabilis* wings; 5. sympatric *C. maculata* females, with sympatric *C. aequabilis* or *C. maculata* wings (Table 1).



**Figure 3.** Female wing types used in the mate choice experiments. Image A (sympatric *C. maculata*) and C (allopatric *C. aequabilis*) display the dark female wing types, and image B (allopatric *C. aequabilis*) displays the light female wing type.

We conducted all trials, consisting of 5 treatments, in 10'x10' (~ 3m x 3m) Coleman screenhouse tents. Each tent contained a 1.2m diameter plastic wading pool with river water submerged vegetation for potential oviposition habitat. Tents were placed immediately next to the 100m stretch of the Au Sable River chosen for the experiments, where there were active male territories and perch sites (Figure 4). The tents

were staked down in corners, and the screen edges were held down with logs or rocks to prevent damselflies from escaping and predators from entering (*e.g.* frogs).



**Figure 4.** Location of the screenhouse tents used for *Calopteryx* mate choice experiments. All tents were set up along a 100m stretch of the Au Sable River in Grayling, MI.

Trials were conducted under ideal weather conditions only (sunny, temperatures above 20°C, and low wind speeds). If females did not require wing grafts, they were collected the morning of the trial. If the females required wing grafts, they followed the procedure as stated previously. Males were collected nearby and placed in each tent after marking them individually with UV-reactive fluorescent powder on their terminal claspers. This powder, either blue, green, orange, pink, or yellow, was transferred to the females upon tandem formation and was used to identify mate choice by individual males. No more than five males, each with a unique color marking, were placed in a tent to ensure independent samples. The males were left to acclimate in the tents for at least 5 minutes before females were added. The number of females varied between 4–10 in each tent, depending on availability, but there were always equal numbers of each female type (*e.g.* dark and light wings), and all females within each tent were treated the same (all manipulated or all unmanipulated). The damselflies were left to mate during active

*Calopteryx* mating times (~1000 – 1900 hrs), for an average of nine hours and then collected from the tents and stored in the freezer in individual glassine envelopes until analyzed.

To compare body and wing differences between the female types in each treatment, all females used in the tents were imaged using a Doxie® flatbed scanner with a ruler before analysis. The damselflies were placed flat on their back in the scanner, with wings spread and held in place using small rectangular glass pieces.

### *Tether Experiments*

To determine the mating preferences of *Calopteryx* males in a more natural setting than tents, we also conducted tether experiments in the streams at both allopatric and sympatric populations. Tether experiments were conducted at three locations: Au Sable River, Bertrand Creek, and Smith Creek (Figure 1). In each trial, two manipulated females, with either light or dark wings, were presented to *Calopteryx* males in the stream (Figure 3). We collected females from the experimental sites and the wings were manipulated in the same way as the tent experiments (Appendix A, Figure 2). Allopatric *C. aequabilis* males were given one treatment, allopatric *C. maculata* males were given two treatments, and sympatric *Calopteryx* males were given four treatments (Table 1).

We tethered the females at the base of their abdomen using a slip knot made of nylon light-weight fishing line (SpiderWire Ultimate-Mono 10lb), a common method used in damselfly mating behavior studies (Miller and Fincke, 1999; Andrés et al., 2002; Iserbyt and Van Gossum, 2011; Schultz and Fincke, 2013). Female pairs were used in no more than three trials to avoid unintentional bias. Each female was tethered to the center of a floating platform comprised of a Nymphaeaceae (water lily) or *Platanus occidentalis* (sycamore) leaf on top of a Styrofoam block. We anchored the platforms to nearby sticks, branches, or plants in the stream using the lightweight fishing line. In each trial, platforms were placed approximately 20cm apart in active mating sites to ensure both female options were visible to males. Sites were selected based on at least one observed *Calopteryx* male guarding a territory, and an abundance of plants for oviposition. To video record each trial, we placed a camera (Nikon Coolpix W100) at standard settings about one meter away from the tether platforms on a tripod.



Trials began after the males returned to activity in the mating site following setup. Each trial lasted approximately five minutes. At the start of each trial, we recorded the total number of damselflies within a 3m radius, recording species and sex. All trials were observed from five meters away. For each trial, we quantified three mating behaviors, including the total number of courtship attempts (males “dancing” or displaying his wings to a female), the number of mating attempts (males attempting to clasp a female around the prothorax), and the number of tandem formations (males who successfully clasped a female around the prothorax). All mating sites were at least 10m apart, and sites were not used more than once. To ensure independent male choice measurements, the second male who responded to the trial was counted in the analysis only if the first male was observed leaving the mating site or if it was collected.

### *Species Comparisons*

To better understand the composition of the sympatric *Calopteryx* populations at our study sites, we determined the ratio of *C. aequabilis* to *C. maculata* through visual encounter surveys. We conducted surveys from June 30<sup>th</sup> to July 7<sup>th</sup>, 2018 along a 100m stretch of the Au Sable River. Surveys were conducted between 800 – 1800hrs at the same location. Surveys lasted approximately 15min and were conducted by two observers, multiple times per day. The abundance of each species and sex was recorded, as well as the temperature at the time of each survey using a Kestrel 3000 Weather Meter.

To verify observed differences between *Calopteryx* females, we compared body length, wing length, and wing intensity. Females from allopatric and sympatric populations were scanned using a portable flatbed scanner with a scaling ruler attached. The damselflies were placed flat on their back and held in place using small rectangular glass pieces. All scanned females were measured in terms of body length, wing length, and wing intensity in ImageJ. Body length measured from the center of the thorax to the tip of the abdomen. Wing length measured from the connection point on the thorax to the furthest tip of the wing. Wing intensity was the greyscale measure of a random ~0.25 x 0.25cm area of each wing. For each female, wing length and wing intensity was an average of all four wings.

**Table 1.** Treatments of dark and light winged *Calopteryx* females given as potential mates to *C. aequabilis* (CA) and *C. maculata* (CM) males from allopatric (allo) or sympatric (sym) populations. Treatments indicate if they were used in the tent experiments, tether experiments, or both. Female body type indicates the population and species of the females used in the mate choice experiments. Female wing type indicates the population and species of the female wing source for the grafting procedure. If no wing grafts were used, wing type was labeled as not manipulated (NM). All dark-winged females are listed first in each treatment.

Treatment	Female Body Type	Female Wing Type	Differed in Body Type	Differed in Wing Type	Differed in Wing Intensity	Males Tested
1 (tent)	sym CM	NM	✓	✓	✓	sym CM/CA
	sym CA	NM				
2 (tent)	sym CA	sym CM	✓	✓	✓	sym CM
	sym CM	sym CA				
3 (tent/tether)	sym CM	sym CM	X	✓	✓	sym CM/CA
		sym CA				
4 (tent/tether)	sym CA	allo CA	X	X	✓	sym CM/CA
		sym CA				
5 (tent/tether)	sym CM	allo CA	X	X	✓	sym CM/CA
		sym CA				
6 (tether)	sym CA	sym CM	X	✓	✓	sym CM/CA
		sym CA				
A1 (tether)	allo CA	allo CA	X	X	✓	allo CA
		sym CA				
M1 (tether)	allo CM	allo CA	X	X	✓	allo CM
		sym CA				
M2 (tether)	allo CM	sym CM	X	✓	✓	allo CM
		sym CA				

### *Data Analysis*

To measure mate preferences in the tent experiment, I recorded the transfer of fluorescent powder from males to females during tandem formation. While tandems do not always lead to successful copulation in *Calopteryx* (Oppenhiemer and Waage, 1987), they do show intent to mate (Battin, 1993) and therefore indicate male mating preference. The fluorescent powder transferred to females was easily visible using a UV light in a dark room or dark box, even after females were stored in the freezer. Since each male was marked with a unique color, the specific male that formed a tandem could be determined. Of the total number of males who mated in all tents of the same treatment, the proportion of males preferring dark-winged females was determined. A binomial test for each treatment and combined treatments were conducted in R-3.5.3. Males were excluded from the analysis if no preference was shown, meaning the male did not attempt to mate, as our goal was to measure preference relative to female wing pigmentation, not male mating inclination. Additionally, all males showing preference for both wing types were noted in the results, but not included in the binomial test do to the restriction of the test.

To measure mating preferences in the tether experiments, any courtship, mating attempt, or tandem formation by males for a specific female type was used as an indication of male mating preference. Due to small sample size, all tether treatments were combined, so no female type differences were measured. Similar to the tent experiments, males were excluded from the tether analysis if no preference or preference for both wing types were shown. We conducted Fisher's Exact Tests in R-3.5.3 to compare the wing preferences of *C. aequabilis* and *C. maculata* from allopatric and sympatric populations

Species and sex ratios along the Au Sable River were determine by averaging the all survey abundances in terms of species and sex. We compared the average total abundance of *C. aequabilis* and *C. maculata* using two-tailed t tests in R-3.5.3. Abundances were also compared between *C. aequabilis* and *C. maculata* in terms of males and females.

Female body and wing comparisons were made using two-tailed t-test in R-3.5.3. We excluded females from the analysis if accurate measurement could not be made (*i.e.*

female was damaged). Average body length and wing length was compared between *C. aequabilis* and *C. maculata* females. Additionally, wing intensity was compared between allopatric *C. aequabilis*, sympatric *C. aequabilis*, and sympatric *C. maculata* females. A Levine's test of equal variance was also run to compared female wing intensity variance between allopatric and sympatric *C. aequabilis* populations.

## Results

### *Tent Experiments*












None of the 30 sympatric *C. aequabilis* males used in the tent experiments formed tandems with females. However, of the 168 sympatric *C. maculata* males in the tent study, 47 showed a preference for either dark- or light-winged females. Because our aim was to measure male preference of dark versus light-winged females, and not overall mating inclination, we show the results of males who indicated a single preference. However, the proportion of males who responded in each treatment ranged from 24% to 39% but were not statistically different between any treatment, including between the treatment with unmanipulated females and any of the treatments with manipulated females (pairwise Z-tests:  $z > 1.8$ ,  $p > 0.07$  for all). Two males showed preference for both female types and were therefore excluded from this analysis.

The results of male sympatric *C. maculata* mate choice in the tent experiments are shown in Table 2. The sample sizes of females and males tested in each treatment varied. *C. maculata* males significantly preferred dark-winged females, regardless of whether the female bodies or wings were heterospecific or conspecific. Binomial tests indicate that 87.5% of sympatric *C. maculata* males preferred the unmanipulated sympatric *C. maculata* females over *C. aequabilis* (Treatment 3;  $p = 0.031$ ). Only 2 males responded when given a choice of sympatric females with heterospecific wings (Treatment 2), and although both preferred the dark-winged *C. aequabilis* females with sympatric *C. maculata* wings, the same size was too small to analyze. When given only *C. maculata* females with either dark conspecific or light heterospecific sympatric manipulated wings (Treatment 3), 85.7% of the males preferred the dark-winged females ( $p = 0.006$ ). When given *C. maculata* females with allopatric and sympatric *C. aequabilis* wings (Treatment 4), 75% preferred *C. maculata* females with dark wings ( $p = 0.109$ ). Similarly, 86.7% of

males preferred the dark-winged over light-winged *C. aequabilis* females (Treatment 5;  $p = 0.003$ ).

I also compared *C. maculata* male preference based on female wing type by combining treatments according to female wing type, regardless of species or population type on which the wings were placed. Binomial tests indicate that 87.5% of males significantly preferred females with dark, sympatric *C. maculata* over light, sympatric *C. aequabilis* wings ( $p < 0.001$ ; Table 2). Similarly, 82.6% of males significantly preferred females with dark, allopatric over light, sympatric *C. aequabilis* wings ( $p = 0.001$ ; Table 2). If all treatments are combined, 85.1% of males preferred dark-winged over light-winged females ( $p < 0.001$ ; Table 2).

**Table 2.** Female treatment types in tent experiments with sympatric *C. maculata* males. Females in the tent trials were either *C. maculata* (CM) or *C. aequabilis* (CA). The number of males indicating a choice are shown as  $N_{\text{males}}$ . The p-values (two-tailed for binomial tests for each tent treatment are shown, in which all binomials were tested against the expected proportion of 0.5. P-values (two tailed) of binomial tests for combined treatments according to female wing type are also included.

Treatment	♀ Body Type	♀ Wing Type		♂ Preference Proportion ( $N_{\text{males}}$ )	Treatment Binomial p-value	Total ♀	Total ♂	Combined ♂ Preference Proportion ( $N_{\text{males}}$ )	Combined Binomial p-value
1	CM	Unmanipulated		0.875 (7)	0.031*	49	22		
	CA	Unmanipulated		0.125 (1)					
2	CA	sympatric CM		1.000 (2)	N/A	26	15	0.875 (21) prefer sympatric wings	< 0.001**
	CM	sympatric CA		0.000 (0)					
3	CM	sympatric CM		0.857 (12)	0.006*	49	36		
	CM	sympatric CA		0.143 (2)					
4	CM	allopatric CA		0.750 (6)	0.109	25	28		
	CM	sympatric CA		0.250 (2)					
5	CA	allopatric CA		0.867 (13)	0.003**	64	63	0.826 (19) prefer allopatric wings	0.001**
	CA	sympatric CA		0.133 (2)					

\* indicates a significant p-value with  $\alpha = 0.05$

\*\* indicates power =  $(1 - \beta) > 0.80$

### *Tether Experiments*

The tether experiments included mate choice behavior from both *C. maculata* and *C. aequabilis* males and were analyzed in the same way as the tent experiments, using the percentage of responding males that chose one of two female types. The total number of males showing a single preference were 8 sympatric and 28 allopatric males (Table 3). Four *C. aequabilis* males, one in allopatry and 3 in sympatry, showed preference for both female types and were therefore excluded from the analyses.

Due to the small sample size within each treatment type, all tether treatments were combined to examine male preference for light versus dark wings. These within-treatment sample sizes, however, are reported here with reference to the treatment number of Table 1 in the Methods. In the allopatric *C. aequabilis* population, 14 males responded to the model, with 12 preferring the allopatric *C. aequabilis* females with allopatric *C. aequabilis* wings (Treatment A1). In the allopatric *C. maculata* population, 14 males also responded to the model, with all 14 preferring the dark-winged female type: nine males preferred allopatric *C. maculata* females with allopatric over sympatric *C. aequabilis* wings (Treatment M1) and five males preferred allopatric *C. maculata* females with sympatric *C. maculata* over *C. aequabilis* wings (Treatment M2).

In the sympatric populations, 5 *C. maculata* and 3 *C. aequabilis* males responded to the tether experiments. All *C. maculata* males preferred the dark female wing types: one male preferred *C. maculata* females with sympatric *C. maculata* over *C. aequabilis* wings (Treatment 3), two males preferred *C. aequabilis* females with allopatric over sympatric *C. aequabilis* wings (Treatment 4), and two males preferred sympatric *C. maculata* females with allopatric over sympatric *C. aequabilis* (Treatment 5). In contrast, all three *C. aequabilis* males preferred the light female wing types: two males preferred sympatric *C. aequabilis* females with allopatric over sympatric *C. aequabilis* wings (Treatment 3) and one male preferred sympatric *C. maculata* females with sympatric over allopatric *C. aequabilis* wings (Treatment 5).

Male preferences in the tether experiment are summarized in Table 3. Fisher's exact tests indicate that sympatric and allopatric *C. aequabilis* males differ significantly in female wing preference, with allopatric males preferring dark wings ( $p=0.0147$ ) and sympatric males preferring light wings ( $p=0.0178$ ). This was not the case between

sympatric and allopatric *C. maculata* males, which both prefer dark-winged females ( $p=0.999$ ). The allopatric populations of both *C. aequabilis* and *C. maculata* similarly prefer dark-winged females ( $p=0.4815$ ).

It is important to note that one allopatric and three sympatric *C. aequabilis* males displayed preferences for both female types. The allopatric male first approached the dark-winged female and attempted to form tandem. Due to an alignment issue from wing manipulations, that particular dark-winged female was unable to form tandem with the male. After two mating attempts with the dark-winged female, the male switched his attention to the light-winged female. In the sympatric populations, the three *C. aequabilis* males showed a preference for both dark- and light-winged females in the form of a brief courtships of wing displays. Due to the lack of a distinguishable preference, these males were excluded from the analysis.

### *Species Comparisons*

Population composition surveys along the Au Sable River showed the average abundance of *C. maculata* was significantly greater than *C. aequabilis* ( $df = 27.868$ ,  $t = 5.2086$ ,  $p < 0.0001$ ), occurring at an average ratio of 2.4 *C. maculata* to 1 *C. aequabilis*. When examined just in terms of sex, a significant difference was found between the average abundance of *C. maculata* and *C. aequabilis* males ( $df = 25.825$ ,  $t = 7.1056$ ,  $p < 0.0001$ ), but not females ( $df = 36.528$ ,  $t = 0.1656$ ,  $p = 0.8694$ ; Figure 5).

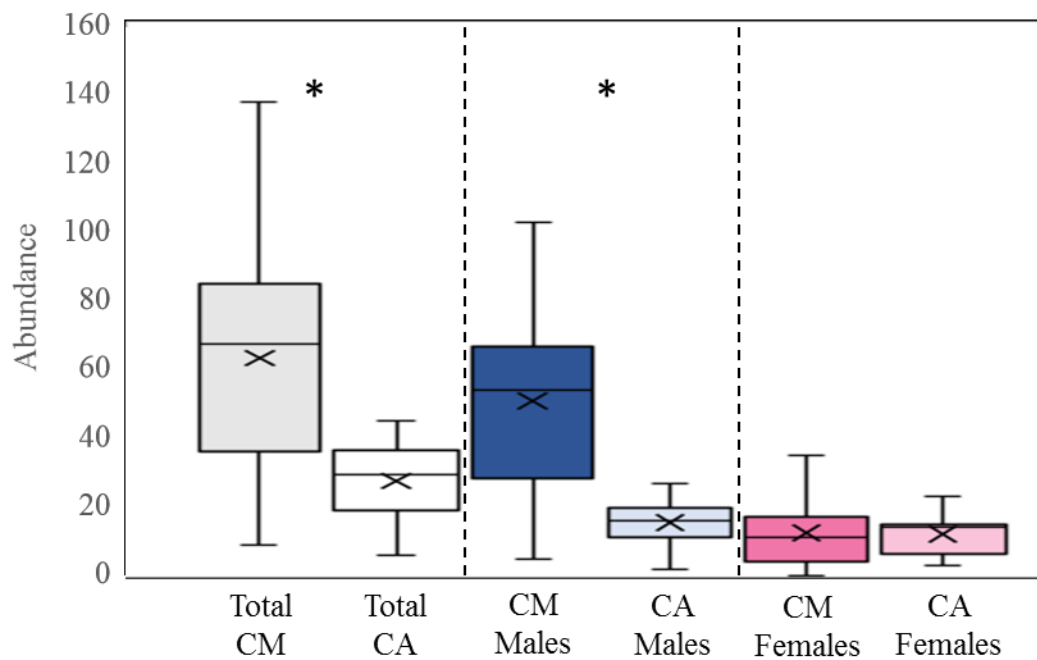
Within females, *C. maculata* have significantly shorter bodies ( $df = 181$ ,  $t = 19.182$ ,  $p < 0.0001$ ) and wings ( $df = 130$ ,  $t = 14.041$ ,  $p < 0.0001$ ; Figure 6). Looking at females by population, wing intensity significantly differed between sympatric *C. aequabilis* and *C. maculata* females ( $df = 150$ ,  $t = 23.454$ ,  $p < 0.0001$ ). Wing intensity quantified as the average greyscale measure of females wings also differed between allopatric *C. aequabilis* females and both sympatric *C. aequabilis* ( $df = 134$ ,  $t = 27.742$ ,  $p < 0.0001$ ) and *C. maculata* ( $df = 96$ ,  $t = 8.548$ ,  $p < 0.0001$ ; Figure 6). The variation in wing intensity between allopatric and sympatric *C. aequabilis* females was also shown to be significantly different. Allopatric *C. aequabilis* had a standard deviation of 14.92, while sympatric *C. aequabilis* had a variation of 6.98 ( $F_{43,40} = 0.219$ ,  $p < 0.0001$ ).



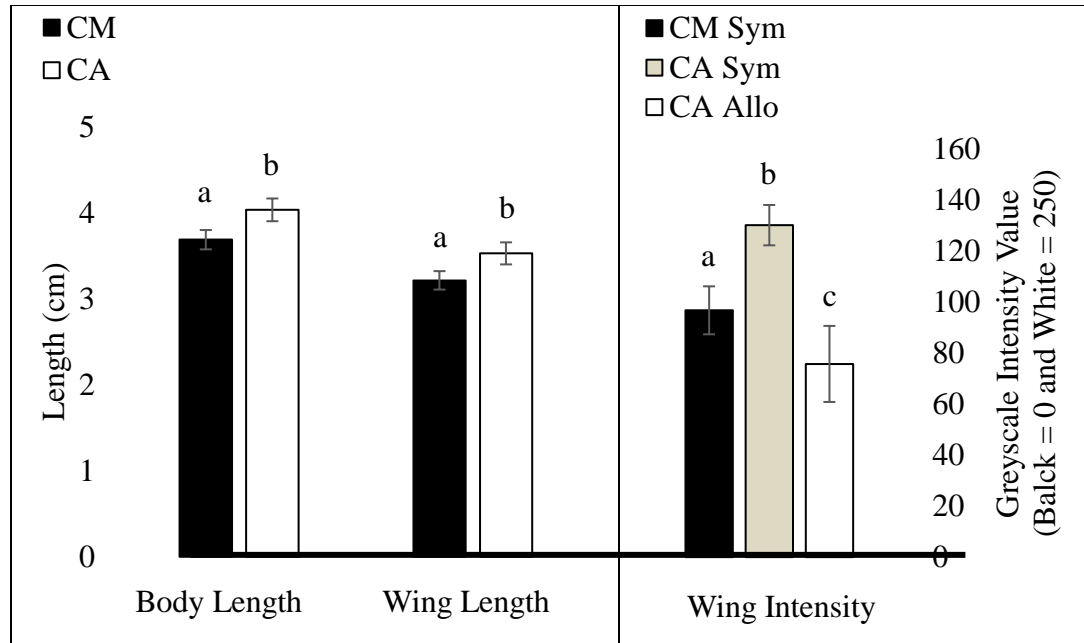
**Table 3.** Number of males responding to female choices in the tether experiments. Population preference ratios are displays in terms of males showing a preference for dark-winged to light-winged females. The p-values are from Fisher's Exact Tests of the male preferences of dark and light wings in four comparisons, between populations and species.

Species	Population Preference (dark wings: light wings)		p-value
	Allopatric	Sympatric	
<i>C. aequabilis</i> Males	12:2	0:3	<b>0.0147*</b>
<i>C. maculata</i> Males	14:0	5:0	0.9999
p-value	0.4815	<b>0.0178*</b>	

\* indicates a significant p-value with  $\alpha = 0.05$



**Figure 5.** Relative abundance of *C. aequabilis* (CA) and *C. maculata* (CM) along the Au Sable River shown in terms of species and sex. The average abundance of each ground is denoted by the X. Significant differences between CA and CM pairs were determined using a two-tailed t test with  $\alpha = 0.05$  are denoted with a star (\*).



**Figure 6.** Average body length, wing length, and wing intensity of *C. maculata* (CM) and *C. aequabilis* (CA) females. Wing Intensity was examined separately in sympatric (Sym) CM, Sym CA, and allopatric (Allo) CA females.

## Discussion

### *Tent Experiments*

To evaluate male mating preferences of female wing color, I first conducted tent experiments in sympatric populations. *C. aequabilis* and *C. maculata* females differ in body and wing size (Figure 6), therefore I manipulated the wing pigmentation by cutting and pasting wings between females. In the tent treatments, I provided males with a choice of two female types to identify whether males showed mate preference, and if so, on what trait in particular. In these trials, only *C. maculata* males behaved naturally and formed tandems with females, while *C. aequabilis* males would not respond. Therefore, the mating preferences of *C. aequabilis* males could not be determined using this method. In all tent treatments, *C. maculata* males consistently showed a preference for dark-winged females, regardless of which species the wings came from and on which species they were glued. Though two males did show a preference for both female types, both males attempted to mate with two dark-winged and one light-winged female (Table 2; Figure 6). Interestingly, males were equally willing to mate with heterospecific females, and they preferred heterospecific females with dark wings from allopatric heterospecifics

(Table 2). This mating behavior indicates that male *C. maculata* would readily court dark-winged heterospecific in sympatric populations, which is presumably a selection pressure that led to the evolution of light-winged *C. aequabilis* females in sympatry.

The conclusion of these tent experiments supports previous results published by Waage (1975), which showed that *C. maculata* males prefer dark-winged females and that *C. aequabilis* males do not respond in mate choice trials in artificial settings. The reason *C. aequabilis* males failed to respond is unknown. There may be behavioral difference between the two *Calopteryx* species. *C. aequabilis* males are very site specific (Conrad & Herman, 1990), and therefore may not have been willing to mate when removed from their territory. In contrast, some *C. maculata* males are not territorial (Waage, 1979a) and perhaps are more willing to respond to females in tents. Additionally, though the mate choice trials by Waage (1975) were not in tents, perhaps *C. aequabilis* males are more discerning about female behavior, as dead females were used.

#### *Tether Experiments*

The goal of the tether experiments was to conduct similar trials outside of a tent, to encourage male *C. aequabilis* to respond within their active mating territories. The method and experiments were successful, as both *C. aequabilis* and *C. maculata* males responded to tethered females (Table 3). The results of the tether experiments supported the prediction that *C. aequabilis* male mating preferences would differ between sympatric and allopatric populations, and their preference would mate local wing color of conspecific females. Both *C. aequabilis* and *C. maculata* males preferred dark-winged females in allopatry, which matches the color of local conspecific females (Table 3; Figure 5). In sympatry however, *C. maculata* males preferred dark-winged females and *C. aequabilis* males preferred light-winged females, which also match the wing pigmentation of local conspecific females (Table 3).

Surprisingly, all the males who showed a preference for both female types and were excluded from the analysis were *C. aequabilis* males. This behavior may hint at a hierarchy of mating behaviors and differences in preference strengths that should be included in future studies.

### *Asymmetries in Reproductive Character Displacement*

Asymmetries in reproductive character displacement exist when one sympatric species shows greater divergence than the other, due to different selection pressure (Cooley, 2007). This asymmetric divergence has been seen in many systems (Smadja & Ganem, 2005; Jaenike et al. 2006; Pfenning & Stewart, 2010), including *C. aequabilis* and *C. maculata*. While sympatric female *C. maculata* show some signs of divergence, it is less pronounced than *C. aequabilis* (Waage, 1975; Waage, 1979b). The reason for this asymmetry is likely due to differences in population abundances that cause different strengths of selection on species recognition. Along the Au Sable River, *C. maculata* are significantly more abundant than *C. aequabilis* (Figure 5). This difference in abundance is specifically due to males and not females, as females of the two species occur along the river at a 1:1 ratio. Males on the other hand, occur at an approximate 4:1 ratio of *C. maculata* to *C. aequabilis*. This difference in male abundance can put a greater pressure on *C. aequabilis* males to identify the correct females because they are more likely to encounter a heterospecific females. Additionally, *C. aequabilis* females are under greater pressure to be accurately recognized, as they are more likely to be harassed by heterospecific males (Waage, 1975). This reasoning is supported by studies on sympatric species, which show that the more abundant species is less influenced by sympatric selection pressures (Cooley, 2007). In this study, both species of males were willing to court and form tandems with heterospecific females (Tables 2 and 3). Future studies could examine the likelihood of interaction that females experience with males of each species, given their relative abundances in the population.

It is interesting to note that the wing intensity variation of allopatric *C. aequabilis* females was significantly greater than the variation in sympatric female *C. aequabilis* (Figure 6). As selection pressures act on the wing color of sympatric *C. aequabilis* females, it is expected that variation of that trait within the population would decrease (Hill & Mulder, 2010).

While coevolution of mating traits and mating preferences has been observed in many systems exhibiting reproductive character displacement (Uy & Borgia, 2000; Schwartz & Hendry, 2007; Grace & Shaw, 2011), little is known about how this displacement arose. Studies are needed to determine if divergence in male preferences

drove changes in female traits, or if a divergence in female traits drove a change in male preferences. Additionally, it is still unknown if these male mating preferences are inherited or learned behaviors. Measurements of male mating preferences throughout a breeding season could help us understand whether male mate choice changes as males learn which females are conspecific. Finally, additional studies are needed to determine the effects of population ratios on selection pressures. Though asymmetries have been observed in this system, direct studies of how species abundances affect selection pressures could help us understand the evolution of species differences.

### *Conclusion*

Through manipulation of female wings in mate choice experiments, this study shows that males use female wing pigmentation for species identification. Divergence of female wing color in sympatric populations led to the hypothesis that reproductive character displacement acts on female wing pigmentation (Waage, 1979b), but this hypothesis was not tested directly in mate choice trials with both species of males. By comparing female wing color preferences of *Calopteryx* males between allopatric and sympatric populations, this study supports our predictions that male mating preferences would correspond to female character displacement. Here we provide direct evidence that reproductive character displacement is acting within this North American *Calopteryx* system, and that both female wing color and male mate preference of sympatric *C. aequabilis* have diverged to improve species recognition.

## Appendix

The wing manipulation procedure was as follows:

1. Wing length of manipulated female were roughly measured using a ruler and recorded.
2. From the wing supply, a new wing set was selected similar in size to the original wing set.
3. Wings from the frozen supply were cut off using a razor blade as close to the body as possible.
4. Live female damselfly was placed on her back with her wings open and flat on the table (Figure 3A).
5. A small weight, such as a microscope slide, was used to hold down the wings of the damselfly being manipulated during the procedure (Figure 3B).
6. One wing was manipulated at a time to ensure the proper forewing or hindwing was attached.
7. To manipulate the wing, a razor blade was used to cut most of the wing off, leaving a small portion of the wing, roughly eight wing cells in length (~4mm).
8. The bottom rows of cells were also removed from the small remaining portion of wing, so that only the top two rows of wings cells remained.
9. A small amount of light-weight UV curing glue was applied to the tip of the new wing.
10. A new wing was then placed over the remaining portion of the damselfly wing, so that the wings cells were precisely aligned (Figure 3C and 3D).
11. The new wings were secured by holding a small UV light directly over the attachment point for approximately 20 seconds (Figure 3E).
12. To minimize the chance of UV radiation to manipulated female, the UV curing light was placed directly over the wings, and not the body.
13. To ensure the new wing was securely attached, the wing was tugged slightly with forceps, then the wing was complete (Figure 3F).
14. Steps 7-13 were repeated for each wing.

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